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Superinfection-Induced Apoptosis and Its Correlation with the Reduction of Viral Progeny in Cells Persistently Infected with Hz-1 Baculovirus

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Differential induction of necrosis or apoptosis was found upon challenge of cells of the insect *Spodoptera frugiperda* productively or persistently infected with Hz-1 baculovirus, respectively. Unlike parental SF9 cells, which were essentially all killed by virally induced necrosis, persistently infected cells underwent a process of massive cell death by apoptosis; cells which were not killed by apoptosis then reestablished a cell monolayer. Upon viral challenge, the yield of viral progeny was reduced greatly in persistently virus-infected cells but not in parental cells. Immunolabelling of individual cells revealed that upon viral challenge, production of viral progeny was detectable only in necrotic cells and not in apoptotic cells. These results indicated that induction of apoptosis greatly reduces the yield of viral progeny in cells persistently infected with Hz-1 baculovirus. This is the first report of apoptosis induction in persistently infected cells upon viral superinfection.

Hz-1 baculovirus is a member of the family *Baculoviridae* and serves as the type species of the subfamily *Nudibaculovirinae* (31). The virus is rod shaped and contains a double-stranded, covalently closed, circular DNA genome of 230 kb (7, 15).

It has been reported that Hz-1 baculovirus can induce both productive and persistent infections in different insect species (4, 8). When the virus infects host cells which have not been previously exposed to it, a productive infection ensues, during which hundreds to thousands of progeny viruses are produced in each cell and the host cell lyses within 12 to 24 h postinfection (h.p.i.) (4, 5, 8, 24). When host cells are killed during Hz-1 baculovirus lytic infection, obvious disruptions of both the cytoplasmic and nuclear membranes plus swelling and damage to mitochondria are observed by electron microscopy (24), indicating cell death by necrosis (17). Cells persistently infected with Hz-1 baculovirus, on the other hand, can be established by infection with a virus that has undergone serial, undiluted viral passages (4, 8). Such persistently infected cells have been reported to be resistant to superinfection with Hz-1 baculovirus (4, 24, 33).

Although Hz-1 baculovirus provides one of the best model systems for studying persistent viral infection (4, 8), very little is known about the association between the virus and host cells during productive and persistent viral infections. To understand these associations, the responses of parental and persistently infected *Spodoptera frugiperda* cells upon viral challenge have been analyzed (28). We found that *S. frugiperda* cells persistently infected with Hz-1 baculovirus are resistant to infection with the same virus but not to infection with a heterologous virus, *Autographa californica* multiple nuclear polyhedrosis virus (Ac*M*NPV). The phenomenon of resistance to the homologous virus was defined as homologous interference.

In this report, we describe several novel discoveries concerning superinfection of *S. frugiperda* cells persistently infected with Hz-1 baculovirus. We found that apoptosis was induced by superinfection with homologous virus of *S. frugiperda* cells which were persistently infected with Hz-1 baculovirus. This is the first discovery of apoptosis induction in persistently virus-infected cells. We also provide evidence showing that induction of apoptosis greatly reduces the yield of viral progeny. Furthermore, we demonstrated that the homologous interference observed in persistently virus-infected *S. frugiperda* cells is achieved through death of a subset of cells and subsequent reestablishment by a surviving subset of cells that is not reported elsewhere in the field of persistent viral infection. These results may provide important insight into the interesting homologous interference phenomenon which is frequently observed in persistently virus-infected eucaryotic cells (2, 12).

MATERIALS AND METHODS

Cells and viruses. SF9 and SF21AE (29) insect cells were grown in modified TNM-FH medium at 26°C (5). Cells were maintained as monolayer or suspension cultures. Cloned persistently infected SFP1 and SFP2 cells were established from SF9 and SF21AE cells, respectively, by the procedure described by Burand and Wood (6). Standard Hz-1 baculovirus was plaque purified from an Hz-1 B1 viral isolate (8). The plaque-purified 1A clone of Ac*M*NPV was used in this study (32). Viral infection was performed as described previously (8).

Identification of necrotic and apoptotic cells. The identification of necrotic and apoptotic cells was based on the morphological changes described and illustrated by Kerr and Harmon (17). Further characterizations of apoptotic cells were assisted by rhodamine 123 (9, 11) and acridine orange staining (10). Staining by rhodamine 123 was performed as follows. Cells were stained with 10 μg of rhodamine 123 per ml at various times postinfection and incubated at room temperature for 30 min. After staining, cells were washed with phosphate-buffered saline (PBS) three times and examined under a fluorescence microscope. For acridine orange staining, cells at various times postinfection were fixed with glacial acetic acid-anhydrous methanol (1:3) at room temperature for 5 min. After fixing, cells were washed twice with PBS containing 0.1% Triton X-100. Cells were then stained with 10 μg of acridine orange per ml in working solution (10 ml of 0.01 M EDTA, 15.5 ml of 1 M NaCl, 31.5 ml of 0.4 M Na_2HPO_4, 18.5 ml of 0.2 M citric acid, 24 ml of distilled H_2O, final pH 6.0) for 30 min at room temperature in darkness. After staining, cells were washed with PBS three times, for 10 min each time, and examined under a fluorescence microscope.

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Detection of DNA fragmentation in agarose gels. Low-molecular-weight DNA was extracted from both mock- and virus-challenged SF9, SFP1, and SFP2 cells. Cells were attached to six-well tissue culture plates at a density of 1.5×10^6 per well. Duplicate wells were pooled for each time point. Chromatin cleavage was studied by a method based on that of Wyllie and Morris (34). At various times postinfection (12, 24, and 36 h.p.i.), both mock- and Hz-1 baculovirus-challenged cells were lysed in 0.7 ml of buffer containing 20 mM Tris, pH 7.5, 4 mM EDTA, and 0.4% Triton X-100. The cell lysate was then centrifuged in a microcentrifuge for 10 min. Nucleic acids in the supernatant were concentrated by precipitation at −70°C in a solution containing equal volumes of isopropanol and 130 mM NaCl. This precipitate was dried under vacuum and dissolved in 10 mM Tris (pH 7.4)–1 mM EDTA–1% sodium dodecyl sulfate. All of the samples were deproteinized by phenol extraction and then ethanol precipitated. One quarter of the resuspended DNA was treated with 1 μl of RNase (2 mg/ml) at 37°C for 30 min. DNA was then electrophoresed in a 1.2% agarose gel at 50 V for 2 h and visualized by ethidium bromide staining. For a positive control, low-molecular-weight DNA was extracted from a hybridoma thymocyte cell line pretreated with dexamethasone for 10 h.

Immunofluorescent staining of virus-infected cells. Cells in a 24-well plate were infected with Hz-1 baculovirus at a multiplicity of infection (m.o.i.) of 5. At 17 h.p.i., cells were fixed by incubation in glacial acetic acid-anhydrous methanol (1:3) solution for 5 min at room temperature, washed three times with PBS–0.1% bovine serum albumin–0.1% Triton X-100, and incubated for 1 h at room temperature with rabbit polyclonal anti-Hz-1 baculovirus antibody diluted 1:1,000 in PBS containing 3% bovine serum albumin. After three washes with PBS–0.1% bovine serum albumin–0.1% Triton X-100, a fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (heavy and light chains; Zymed Laboratories, South San Francisco, Calif.) was added at a dilution of 1:100 in PBS containing 10% normal goat serum and incubated for 1 h. Each well was then washed three times with PBS–0.1% bovine serum albumin–0.1% Triton X-100 and visualized with a fluorescence microscope. Photographs were taken with the same exposure time by using Polaroid 667 ASA 3200 film.

RESULTS

Differential host responses upon viral challenge. Two persistently infected cell lines, SFP1 and SFP2, were established by Hz-1 baculovirus infection of parental SF9 and SF21AE insect cells, respectively, by a previously described method (8). These two persistently infected cell lines, together with parental SF9 cells, were challenged with an Hz-1 baculovirus inoculum. It was found that the typical response of parental SF9 cells was necrosis (Fig. 1). Surprisingly, the response of SFP1 and SFP2 cells to Hz-1 baculovirus superinfection was apoptosis (Fig. 1). Infected SF9 and SFP1 cells were stained with rhodamine 123, a dye which fluoresces in intact energized mitochondria. During the course of apoptosis, intact energized mitochondria have been reported to be found in apoptotic cells and bodies but not in necrotic cell remains (9, 11). Very few apoptotic cells were found in virus-infected parental SF9 cells (Fig. 1A, SF9-17h.a and SF9-17h.b). However, many apoptotic bodies were observed in superinfected SFP1 cells (Fig. 1A, SFP1-17h.a and SFP1-17h.b). Necrotic cell remains were observed mainly in virus-infected SF9 cells and were not stainable by rhodamine 123 (Fig. 1A, SF9-17h.a and SF9-17h.b). Soon after viral challenge (17 h.p.i.), some necrotic cells were already observed in parental SF9 cells (Fig. 1A, SF9-17h.a and SF9-17h.b; Fig. 1B, SF9-17h). Within the same time frame, however, much more extensive mortality due to apoptosis was observed in persistently infected cells after viral challenge (Fig. 1A, SFP1-17h.a and SFP1-17h.b; Fig. 1B, SFP1-17h and SFP2-17h).

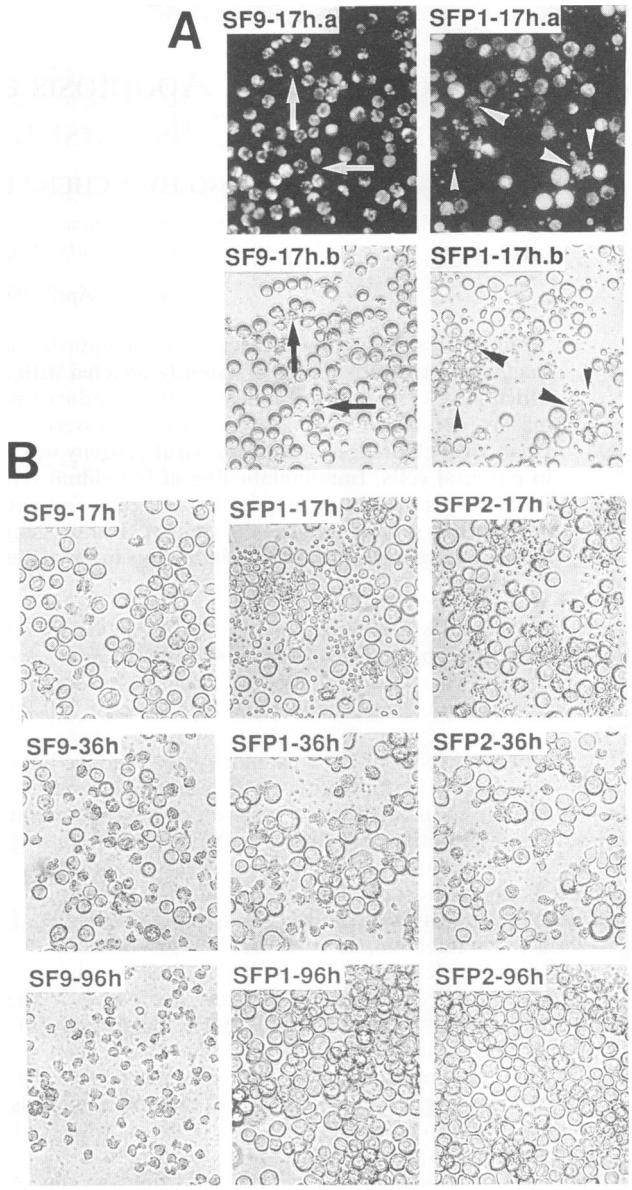


FIG. 1. Appearance of cell cultures at various times after the challenge with Hz-1 baculovirus. Parental SF9 cells and persistently infected SFP1 and SFP2 cells were inoculated with Hz-1 baculovirus at an m.o.i. of 5 and then either subjected to rhodamine 123 staining as described previously (9, 11) or left unstained. (A) Rhodamine 123-stained SF9 and SFP1 cells at 17 h.p.i. (SF9-17h.a and SFP1-17h.a) and bright-field pictures of SF9 and SFP1 cells at 17 h.p.i. (SF9-17h.b and SFP1-17h.b). Panels SF9-17h.a and SF9-17h.b show the same field; similarly, panels SFP1-17h.a and SFP1-17h.b show the same field. Necrotic cells were not stainable with rhodamine 123, as indicated by the arrows (SF9-17h.a and SF9-17h.b); apoptotic cells and apoptotic bodies could be stained and are indicated by the large and small arrowheads, respectively (SFP1-17h.a and SFP1-17h.b). (B) Bright-field pictures of SF9, SFP1, and SFP2 cells taken separately at 17, 36, and 96 h.p.i.

Intensive apoptosis upon superinfection of persistently in-

fected cells. Similar significant levels of virus-induced mortality were observed in both parental (Fig. 1B, SF9-36h) and persistently infected (Fig. 1B, SFP1-36h and SFP2-36h) cells at 36 h.p.i. Interestingly, by 96 h.p.i., most of the SF9 cells had died from necrosis (Fig. 1B, SF9-96h), whereas persistently infected cells which survived from the initial viral superinfection continued to propagate (Fig. 1B, SFP1-96h and SFP2-96h).

The responses of SF9, SFP1, and SFP2 cells to viral challenge were traced through time (Fig. 2). Death of SF9 cells typically began at 13 h.p.i. and increased gradually thereafter. Most of the SF9 cells died from apparent necrosis (Fig. 2A), which is a typical consequence of successful viral infection and replication, as shown previously by transmission electron micrographs of infected cells (4, 24, 33). Less than 4% of these cells died through apoptosis (Fig. 2B). In contrast, most of the superinfected SFP1 and SFP2 cells died from apoptosis. Apoptosis was first observed at 6 h.p.i., and the percentage of apoptotic cells increased drastically thereafter up to 24 h.p.i. and then levelled off gradually (Fig. 2B).

The percentages of the three lines of cells that survived and/or propagated after superinfection were also recorded (Fig. 2C). The proportion of surviving cells in all three lines decreased rapidly after the viral challenge. Notably, the decrease in the number of surviving cells of the two persistently infected cell lines occurred at a much faster rate than that in the number of surviving parental SF9 cells. The decrease in the number of surviving SF9 cells, although occurring more slowly, proceeded steadily and continuously. At 96 h.p.i., essentially no surviving SF9 cells were observed. For the more rapidly dying SFP1 and SFP2 cells, however, the rate of death was arrested at 24 h.p.i. Thereafter, the cells which survived the initial viral superinfection began to grow (Fig. 2C). Thus, viral homologous interference in persistently infected cells is achieved through a process that combines both apoptotic cell death and reestablishment of the host cell population by a subset of surviving cells.

Detection of low-molecular-weight DNAs by viral infection. To determine whether the nucleosomal-length DNA ladders, usually a diagnostic feature of apoptosis (35), were generated in persistently infected cells, low-molecular-weight DNAs were purified from both mock- and virus-infected parental and persistently infected cells. After agarose gel electrophoresis, DNA ladders were visible in persistently infected cells at 12 h.p.i. and could still be observed through 36 h.p.i. (Fig. 3, lanes 8 to 10 and 12 to 14) but not in mock-infected cells (Fig. 3, lanes 3, 7, and 11) or virus-infected SF9 cells (Fig. 3, lanes 4 to 6). Low-molecular-weight DNA was not evident in SF9 cells at 12 h.p.i. (Fig. 3, lane 4). At 24 and 36 h.p.i., randomly degraded DNA fragments were observed in virus-infected SF9 cells (Fig. 3, lanes 5 and 6). Such random degradation of chromosomal DNA has been reported to be the result of cell necrosis (17).

Production of viral progeny is greatly reduced in apoptotic cells. The results of Fig. 2 and 3 collectively suggest that apoptosis occurs so early in persistently infected cells that it may reduce the yield of viral progeny. To test this possibility, both the infectivity and the progeny propagation capability of Hz-1 baculovirus in parental and persistently infected cells were tested by two different methods. In the first method, cells were infected directly with the same dosage of Hz-1 baculovirus and the numbers of plaques formed in these cells were calculated. Numbers of plaques were then plotted as individual bars to represent different cell lines. The results showed that significantly fewer plaques were detected in persistently infected cells than in SF9 cells (Fig. 4A). Our second method estimated the yield of viruses produced from different types of cells, giving a titer of released virus. The numbers of viruses

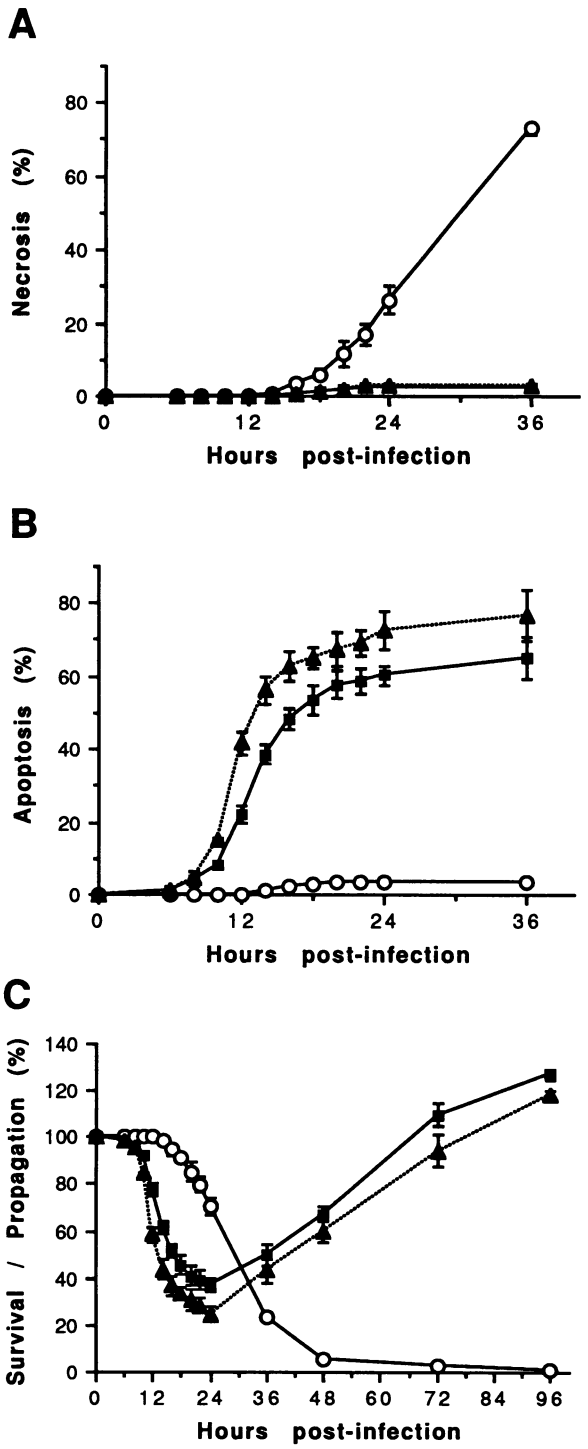


FIG. 2. Cell responses at various times after Hz-1 baculovirus infection. Parental and persistently infected cells were challenged with virus. After viral challenge, numbers of necrotic (A), apoptotic (B), and surviving and propagating (C) cells were calculated as percentages of the cell numbers before challenge. Hz-1 baculovirus at an m.o.i. of 5 was used to infect SF9, SFP1, and SFP2 cells. Percentages of necrosis and apoptosis were calculated each time from 200 originally input parental or persistently infected cells. Data (means ± standard deviations) were collected from four independent experiments. Symbols: ○, SF9; ■, SFP1; ▲, SFP2.

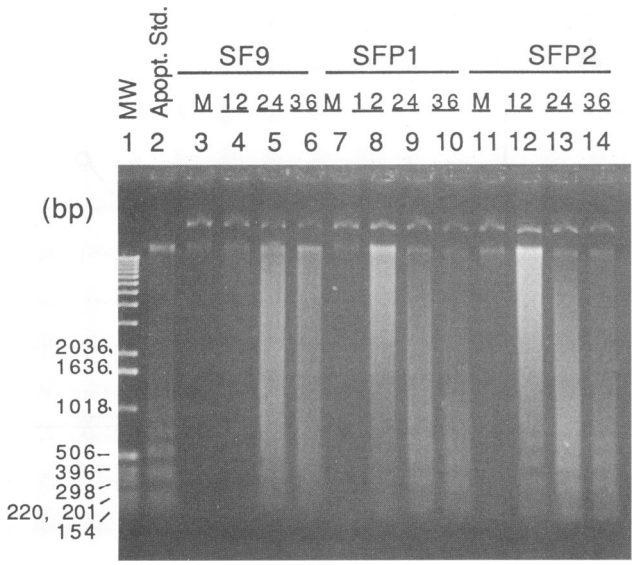


FIG. 3. Agarose gel electrophoresis patterns of low-molecular-weight DNA resulting from Hz-1 baculovirus (m.o.i., 5) infection. Lanes: 1, 1-kb molecular size (MW) markers (Bethesda Research Laboratories, Inc., Gaithersburg, Md.); 2, DNA ladders extracted from apoptotic human T-cell hybridomas (Apopt. Std.) treated with dexamethasone for 10 h and used as a positive control (34); 3 to 14, low-molecular-weight DNAs extracted from Hz-1 baculovirus-infected SF9, SFP1, and SFP2 cells (lane 3, mock-infected SF9 cell; lanes 4 to 6, virus-infected SF9 cells at 12, 24, and 36 h.p.i., respectively; lane 7, mock-infected SFP1 cells; lanes 8 to 10, virus-superinfected SFP1 cells at 12, 24, and 36 h.p.i., respectively; lane 11, mock-infected SFP2 cells; lanes 12 to 14, virus-superinfected SFP2 cells at 12, 24, and 36 h.p.i., respectively). In lanes 3 to 14, low-molecular-weight DNAs were carefully purified from the same number of cells (3×10^6) and a quarter of the extracted DNA was loaded onto a 1.2% agarose gel.

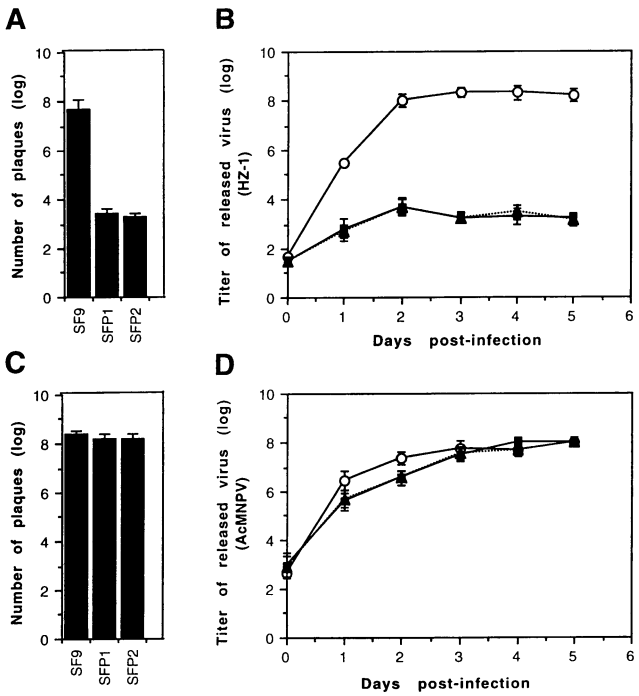


FIG. 4. Viral resistance evaluation of different cell lines. The experiments were divided into two parts. In the first part, sensitivities of different cell lines to direct challenge infection with either Hz-1 baculovirus (A) or Ac*M*NPV (C) were estimated by the plaque assay (28). Hz-1 baculovirus with a titer of 8×10^7 PFU/ml (A) and Ac*M*NPV with a titer of 3×10^8 PFU/ml (C) were serially diluted 10-fold and then used to infect SF9, SFP1, and SFP2 cells directly. The plaque formation capabilities of different cell lines upon virus infection were estimated at 5 days postinfection. In the second part, equal numbers (2×10^5) of SF9, SFP1, and SFP2 cells were first infected with either Hz-1 baculovirus (B) or Ac*M*NPV (D) at an m.o.i. of 0.1. Media were harvested daily for 5 days, and their virus titers were estimated by plaque assay with SF21AE cells. Data (means ± standard deviations) were collected from four experiments with three independent plaque assays. Symbols: ○, SF9; ■, SFP1; ▲, SFP2.

released from superinfected SFP1 and SFP2 cells were decreased 10^4- to 10^5-fold compared with those released from parental SF9 cells (Fig. 4B).

For comparison, a heterologous virus, Ac*M*NPV, belonging to a different subfamily, *Eubaculovirinae* (31), was also used to infect SF9, SFP1, and SFP2 cells. No significant differences were found among the three cell lines either in resistance to Ac*M*NPV infection (Fig. 4C) or in the viruses released from these cells upon viral superinfection (Fig. 4D).

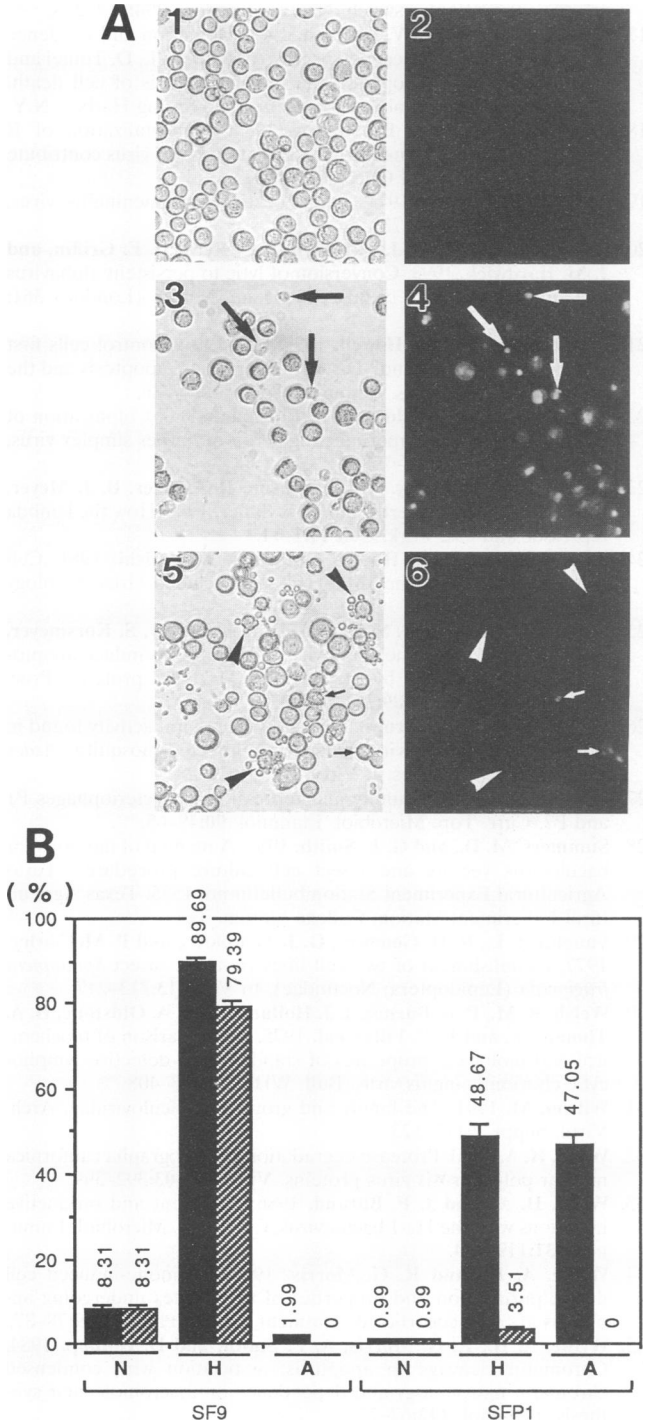
The aforementioned results indicated that overall production of Hz-1 baculovirus in persistently infected cells was much less than that in parental cells upon viral challenge. It is interesting and important to know whether the small amounts of viruses detected in superinfected persistently infected cells were generated from apoptotic cells or from necrotic cells which were present in only a small percentage of the superinfected population. To differentiate these two possibilities, we used fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G and rabbit polyclonal antibody against the virion of Hz-1 baculovirus to label both parental and persistently infected cells at 17 h.p.i. (Fig. 5).

Our results showed that the fluorescein isothiocyanate-conjugated antibody did not label mock-infected cells (Fig. 5A, 1 and 2), indicating that the antibody did not cross-react with host cells. The fluorescein isothiocyanate-conjugated antibody did strongly label SF9 cells productively infected with the virus. Essentially all of the necrotic cells were labelled (Fig. 5A, 3 and 4, arrows; Fig. 5B, column SF9-N). We also observed that many healthy-looking cells were labelled. Presumably, these were infected cells at stages before cell lysis (Fig. 5A, 3 and 4; Fig. 5B, column SF9-H). In contrast, much less intensive antibody labelling was detected in persistently infected cells that were superinfected with virus. Careful examination of the labelled cells revealed that apoptotic bodies were not labelled (Fig. 5A, 5 and 6, big arrowheads; Fig. 5B, column SFP1-A). Instead, only necrotic and a few healthy-looking cells were labelled (Fig. 5A, 5 and 6, small arrows; Fig. 5B, columns SFP1-N and SFP1-H).

DISCUSSION

In this report, we have described how parental and persistently infected cells responded differently to viral challenge. The response of parental cells was necrosis, whereas that of persistently infected cells was apoptosis. When parental cells were infected with the virus, hundreds of viral progeny were produced and the host cells were eventually lysed. Virus-induced cell lysis was observed beginning at 13 h.p.i. and increased slowly (Fig. 2A). Low-molecular-weight agarose gel electrophoresis analysis of parental and persistently infected cells after viral challenge also revealed that DNA degradation in parental cells took place much later than the onset of apoptosis in persistently infected cells (Fig. 3). This relatively

slow host response provides sufficient time for the virus to propagate in parental cells, as revealed by electron microscopy (24). On the other hand, superinfection induced apoptosis in persistently infected cells beginning at 6 h.p.i. and the proportion of apoptotic cells increased drastically thereafter (Fig. 2B). Such quick cell death provided limited time and opportunity for the virus to replicate, and the yield of viral progeny was greatly reduced. Antibody labelling of persistently infected cells which were challenged with Hz-1 baculovirus also revealed that the apoptotic cells did not produce observable amounts of viral progeny (Fig. 5).



As a necessary control, another virus of the same family, Ac*M*NPV, was used to challenge SF9, SFP1, and SFP2 cells. Ac*M*NPV has been reported to carry a gene which blocks virus-induced apoptosis in SF-21 cells (11). As predicted, apoptosis was not evident after Ac*M*NPV infection of cells that were or were not persistently infected with Hz-1 baculovirus (data not shown). In addition, the titers of the viruses produced from parental cells and cells persistently infected with Hz-1 baculovirus differed only slightly. These results indicate that susceptibility to infection with a heterologous virus, Ac*M*NPV, is not significantly altered in cells persistently infected with Hz-1 baculovirus.

Collectively, our findings suggest that production of viral progeny is greatly reduced by superinfection-induced apoptosis in *S. frugiperda* cells persistently infected with Hz-1 baculovirus. Ac*M*NPV with a mutation in the p35 gene has been reported to induce apoptosis in healthy SF21AE cells (11). In SF21 cells, deletion of the p35 gene reduced yields of extracellular, budded virus up to 15,000-fold. In contrast, inactivation of the p35 gene had no effect on virus growth in cultured *Trichoplusia ni* (TN368) cells (14). Superinfection-induced apoptosis in cells persistently infected with Hz-1 baculovirus was also observed in a cell line-specific manner. Superinfection-induced apoptosis was observed only in persistently virus-infected cells originally derived from SF9 or SF21 cells but not in those derived from TN368 cells (data not shown). Clem et al. (11) proposed that cellular apoptotic responses during viral infection probably have significant effects on viral pathogenesis. Similarly, Martz and Howell (21) have proposed that apoptosis evolved as a primitive viral defense response in animals lacking humoral immune systems. Therefore, induction of apoptosis in *S. frugiperda* cells persistently infected with Hz-1 baculovirus is likely to play a role in viral homologous interference.

Levine et al. (20) provided the interesting finding that lytic alphavirus infection can be converted into persistent infection by introduction and expression of the cellular oncogene *bcl-2*. *bcl-2* and other genes which may control the occurrence of apoptosis in insects are poorly understood. However, our findings indicate that regardless of what mechanisms may operate to prevent the death of *S. frugiperda* cells during persistent viral infection, intensive apoptosis can still be triggered upon viral superinfection. It is not clear what mechanism(s) operates this apoptosis induction. It is possible that persistently infected *S. frugiperda* cells express an apoptosis inhibitor and superinfecting virus can somehow interfere with the activity of this apoptosis inhibitor. Rao et al. (25) reported that the adenovirus E1A product induces apoptosis, whereas the viral E1B product or cellular *bcl-2* inhibits apoptosis.

FIG. 5. Immunofluorescent labelling of virus-infected cells. Cells were grown in 24-well plates and infected with Hz-1 baculovirus. At 17 h.p.i., cells were immunofluorescence labelled with antibody against Hz-1 baculovirus. (A) Panels: 1 and 2, mock-infected SF9 cells; 3 and 4, Hz-1 baculovirus-infected SF9 cells; 5 and 6, Hz-1 baculovirus-infected SFP1 cells. 1, 3, and 5: bright-field images of infected cells; 2, 4, and 6, fluorescent images of infected cells. The large arrows indicate necrotic SF9 cells (3 and 4). The small arrows indicate necrotic or nonapoptotic SFP1 cells which were stainable by the fluorescent antibody (5 and 6). The arrowheads indicate apoptotic SFP1 cells which were not stainable by the fluorescent antibody (5 and 6). (B) Vertical bars showing percentages of antibody-stained necrotic (N), healthy-looking (H), and apoptotic (A) cells. Filled bars indicate percentages of unstained cells, and hatched bars indicate stained cells.

Similarly, if an apoptosis inhibitor specific to Hz-1 baculovirus infection is expressed only in parental *S. frugiperda* cells and not in cells persistently infected with virus because of an unknown mechanism, viral gene expression can thus trigger apoptosis in persistently infected cells upon viral superinfection. In any case, further experiments are necessary to elucidate such an interaction between the virus and its host cell.

When a host cell is persistently infected with a virus, it often becomes resistant to superinfection by the same virus, a phenomenon known as viral homologous interference (2, 12). Naturally occurring viral homologous interference in prokaryotic systems has been well characterized (16, 23, 27). Although similar phenomena are intensively studied in the eucaryotic system, relatively little is known (1, 3, 12, 13, 18, 19, 22, 26, 30). In *S. frugiperda* cells persistently infected with Hz-1 baculovirus, we have demonstrated for the first time that apoptosis is induced upon viral superinfection. We also present evidence showing that the yield of viral progeny is greatly reduced once apoptosis is induced. Whether triggering of apoptosis is necessary for viral homologous interference in *S. frugiperda* cells is an important issue for further investigation.

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